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(54) Title: USE OF ANIMAL-DERIVED ANTI-MICROBIAL PEPTIDES FOR CONTROL OF PLANT PATHOGENS

(57) Abstract

    The present invention is directed to the application of an animal-derived Anti-Microbial Peptide ("AMP") to the control of microbes associated with plant disease. Specifically, it has been found that the cytotoxic effects of AMP's, such as defensins and/or magainins, can be successfully used to limit the growth of bacterial and fungal pathogens associated with plant diseases.

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DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

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Description

Use of Animal-Derived Anti-Microbial
Peptides for Control of Plant Pathogens

Technical Field

This invention relates to the use of animal-
derived anti-microbial peptides in the control of
plant-associated pathogens. More particularly, the
invention relates to the use of peptides such as
magainins and defensins in the control of microbes
associated with plant disease.

Background of the Invention

Plant-associated microbial pathogens, such as
various species of bacteria, fungi, and viruses, are
responsible for significant losses in agriculture. The
identification of broad spectrum agents against these
microorganisms may provide enhanced strategies for
plant and crop protection. Thus, the development of
broad spectrum, anti-microbial agents effective against
growth of plant-associated pathogens is desired.

Moreover, there is an ever-present need for new
and better ways to improve agricultural production
using natural agents, or compositions based upon
natural agents, rather than man-made chemicals. Thus,
the identification of natural agents capable of
inhibitory activity against microbial plant pathogens,
termed "plant-associated pathogens" herein, is desired.
Particularly desired is the identification of natural
agents which each display antimicrobial activity
against at least two or more plant pathogen species,
and especially, such agents which each display broad
spectrum antimicrobial activity against different
classes of plant pathogens. By the present invention,
a new class of agents are provided for the control of a
wide range of microbial plant-associated diseases.

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It was also considered desirable to apply recombinant deoxyribonucleic acid (rDNA) and related technologies to provide for the production of such compounds _in vivo_ to allow the modified plant to defend

5   itself against plant-associated pathogens.

Proceeding from the seminal work of Cohen & Boyer, U.S. Patent No. 4,237,224, rDNA technology has become useful to provide novel DNA sequences and produce heterologous proteins in transformed cell cultures. In

10   general, the joining of DNA from different organisms relies on the excision of DNA sequences using restriction endonucleases. These enzymes are used to cut donor DNA at very specific locations, resulting in gene fragments which contain the DNA sequences of

15   interest. Alternatively, structural genes coding for desired peptides and regulatory control sequences of interest can now be produced synthetically to form such DNA Fragments.

These DNA fragments usually contain short single-

20   stranded tails at each end, termed "sticky-ends". These sticky-ended fragments can then be ligated to complementary fragments in expression vehicles which have been prepared, e.g., by digestion with the same restriction endonucleases. Having created an

25   expression vector which contains the structural gene of interest in proper orientation with the control elements, one can use this vector to transform host cells and express the desired gene product with the cellular machinery available. Recombinant DNA

30   technology provides the opportunity for modifying plants to allow the expression of alien antimicrobial peptides.

However, while the general methods are easy to summarize, the construction of an expression vector

35   containing a desired structural gene is a difficult process and the successful expression of the desired

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gene product in significant amounts while retaining its
biological activity is not readily predictable.
Frequently, animal-derived gene products are not
biologically active when expressed in plant systems.

5   To successfully modify plants using rDNA, one must
usually modify the naturally occurring plant cell in a
manner in which the cell can be used to generate a
plant which retains the modification. Even in
successful cases, it is often essential that the

10   modification be subject to regulation. That is, it is
desirable that the particular gene be regulated as to
the differentiation of the cells and maturation of the
plant tissue. In the case of anti-microbial compounds,
it is also important that the modification be performed

15   at a site where the product will be directed to contact
the plant-associated pathogens of interest. Thus
genetic engineering of plants with rDNA presents
substantially increased degrees of difficulty.

       In addition, the need to regenerate plants from

20   the modified cells greatly extends the period of time
before one can establish the utility of the genetic
construct. It is also important to establish that the
particular constructs will be useful in a variety of
different plant species. Furthermore, one may wish to

25   localize the expression of the particular construct in
specific cell types and it is desirable that the
genetically modified plant retain the modification
through a number of generations.

Brief Description of the Prior Art
30   Magainins are reported in Zasloff, M., Proc. Nat.
Acad. Sci USA 84:5449-5453 (1987) and U.S. Patent No.
4,810,777.
       U.S. Patent Nos. 4,543,252, 4,659,692, and
4,705,777 are a series of related patents describing

35   microbicidal compositions corresponding to defensins.

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Disclosure of the Invention

        This invention relates to the use of animal-
derived anti-microbial agents in the control of plant-
associated pathogens. In one aspect, the invention
comprises a method of limiting growth of a plant-
associated pathogen comprising contacting a plant-
associated pathogen with a plant-associated pathogen-
inhibiting amount of an animal-derived anti-microbial
peptide under growth limiting conditions and allowing
said anti-microbial peptide to act upon said plant-
associated pathogen thereby limiting growth by said
plant-associated pathogen.

        In accordance with another aspect of the subject
invention, a DNA construct is provided which comprises
a transcriptional initiation region functional in
plants joined at its 3' terminus to the 5' terminus of
a DNA sequence which codes for at least a portion of a
signal peptide which is secreted from a plant cell and
an animal-derived anti-microbial peptide.

Detailed Description of the Invention

        The present invention is directed to the
application of an animal-derived Anti-Microbial Peptide
("AMP") to the control of microbes associated with
plant disease. Specifically, it has been found that
the cytotoxic effects of AMP's, such as defensins
and/or magainins, can be successfully used to limit the
growth of bacterial and fungal pathogens associated
with plant diseases.

        Plant diseases have been classified on the basis
of symptoms, causative agents, specific plant organs
infected, diseases involving physiological processes,
diseases of specific crop groups and the physiological
functions of a non-diseased plant. In this final
system, diseases are grouped on the basis of their
effect on one of seven essential functions: food

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storage; hydrolysis and utilization of stored food; absorption of water and nutrients by roots; growth and development of meristems; water translocation in the xylem; photosynthesis; and translocation of foods through the phloem. This system focuses on the disease phenomena rather than the pathogen involved because many different kinds of pathogens can be responsible for a specific disease syndrome. Thus, a broad spectrum pathogen control agent would be valuable to treat affected plants based on the observed symptoms.

The primary agents of plant pathology are fungi, bacteria, viruses and viroids, nematodes, parasitic seed plants and a variety of non-infectious agents. As used herein, plant-associated pathogens include the fungi, bacteria, viruses and viroids which cause disease in plants but have little or no infective potential in animals.

Representative plant-associated pathogens include the following:

Fungi

More plant diseases are caused by fungi than by any other pathogenic agent. The fungi include a vast variety of living organisms which lack chlorophyll and must obtain energy from organic matter. The fungi are grouped into various classes based primarily on the morphology of their reproductive stages. The classes of fungi with plant-associated pathogens include: Plasmodiophoromycetes, Chytridiomycetes, Zygomycetes, Oomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes.

Plasmodiophoromycetes

The zoospores of Plasmodiophoromycetes penetrate root cells of the host and become ameboid, and the plasmodium develops. Only a few species in this class cause disease in cultivated plants. Plasmodiophora

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brassicae, the agent of club root of crucifers, is the best known.

Chytridiomycetes

Chytrid fungi produce motile cells (zoospores) with a single flagellum. Only a few species are economically important as pathogens. A true mycelium is lacking in the species that cause plant disease. One of the best-known disease-causing chytrids is *Synchytrium endobioticum*, the agent of black wart of potato.

Oomycetes

The Oomycetes include many fungi causing destructive plant diseases, most of which are in one order, Peronosporales. Included are the water molds, the downy mildews, and the white blisters (white rusts). *Plasmopara viticola*, the cause of downy mildew of grape, and *Phytophthora infestans*, the cause of late blight of potato and tomato, are examples of Oomycetes. All the plant parasitic species of the class have a filamentous, branched, coenocytic mycelium that grows between or within the plant cells. The downy mildews derive their name from the abundance of mycelial branches (sporangiophores) bearing sporangia which grow out from the stomates of diseased leaves. The species of *Albugo*, causative agents of the white blisters, produce sporangia in compact masses under the host epidermis. A whitish blister results, and when the epidermis is ruptured a powdery white crust is evident.

Zygomycetes

Most of the Zygomycetes are saprobes, living on decaying plant and animal matter. One species, *Rhizopus stolonifer*, is a facultative parasite and can cause a serious rot disease of fleshy plant organs after harvest. Examples are soft rot of sweet potatoes and strawberries.

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Ascomycetes

The Ascomycetes are the sac fungi, so named because the sexual or perfect-state spores are produced in a sac like cell, the ascus. The Ascomycetes are the largest class of fungal pathogens. Included are the powdery mildew fungi, which are obligate parasites.

Other examples of Ascomycetes are _Claviceps purpurea_, causing the ergot disease of cereals; _Monilinia fructicola_, the cause of brown rot of stone fruits; _Venturia inaequalis_, the cause of apple scab; _Ceratocystis ulmi_, the cause of the Dutch elm disease; and _Endothia parasitica_, which has nearly eliminated the American chestnut in North America.

Basidiomycetes

Examples of Basidiomycetes that cause plant disease are the smuts, rusts, and the wood-decay fungi of forest trees. Rust fungi include _Puccinia graminis_, the causative agent of black stem rust of cereal grains; _Cronartium ribicola_, the cause of white pine blister rust; and _Gymnosporangium_ species, which cause the cedar apple and cedar hawthorn rusts. About 4000 species of rust fungi are known; all are obligate parasites.

Most of the economically important smut fungi are parasites of corn, sorghums, the cereal grains and other grasses. In the more destructive diseases, the spores develop in and destroy the flower parts so there is a total loss of grain yield. _Ustilago_ species cause smuts of wheat, barley, corn, and oats. Species of _Tilletia_ cause stinking smut or bunt of wheat.

The wood decay- and root rot-causing fungi of forest trees include _Armillaria mellea_ and numerous species of _Fomes_ and _Polyporus_. All of these fungi produce enormous numbers of basidiospores on gills (lamellae) or in pores or tubes on the underside of large complex fruiting bodies (basidiocarps). The

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basidiocarps are referred to as mushrooms, conks, or
bracket and shelf fungi.

Deuteromycetes

There are several thousand species of fungi that
so far as is known reproduce only by asexual spores,
called conidia. Many Deuteromycetes cause serious leaf
spots and flower blights, as well as fruit rots, stem
cankers, and wilts. Examples include species of
<u>Septoria, Gloeosporium, Diplodia, Alternaria</u>, and
<u>Verticillium</u>. The classification of the Deuteromycetes
is based on the morphology of the conidial states and
the mode of development of the conidia.

Bacteria

Over 100 species of bacteria mainly in five genera
cause disease in hundreds of different species of
flowering plants. Destructive bacterial diseases
affect the major cereal, vegetable, and fruit crops.
None of the bacterial pathogens of plants causes
serious diseases of humans or animals.

Each species of bacterium produces a distinctive
pattern of symptoms on those hosts that it attacks.
The main genera of bacterial plant pathogens, and the
symptoms their infections cause, include:

<u>Agrobacterium</u> species cause crown gall, twig gall,
cane gall and hairy root diseases;

<u>Corynebacterium</u> species cause ring rot, tomato
wilt, fruit spot and fasciation;

<u>Erwinia</u> species cause various forms of blight,
wilt and soft rot;

<u>Pseudomonas</u> species cause leaf spots, olive galls,
banana wilt, lilac blight, canker and bud blast;

<u>Xanthomonas</u> species cause leaf spots, cutting rot,
black venation, bulb rot, citrus canker and walnut
blight;

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Streptomyces species cause potato scab and soil rot of sweet potato; and

Rhizobium species cause root nodules on legumes.

With a few exceptions, most of the bacteria that cause disease in plants are non-spore-forming, rod-shaped, gram-negative cells. Some species give a positive reaction with the Gram stain; these include species of _Streptomyces_ and _Corynebacterium_, a few species of _Bacillus_ that cause soft rots, and those species of _Clostridium_ that can decay plant storage organs under anaerobic conditions. Only the species in the first two genera can form endospores. Rickettsia-like bacteria have been observed in diseased plants; unlike other plant pathogenic bacteria, these have been extremely difficult to grow in culture or have not been isolated.

A number of yellow-type diseases previously considered to be caused by viruses have now been shown to be caused by mycoplasma-like organisms (MPLO), members of the Mollicutes. Pleomorphic MPLOs lacking cell walls have been observed in vascular tissues of yellow-affected plants; however, some have not as yet been characterized through isolation and cultivation techniques. The MPLOs cultured from plants affected by the corn stunt and citrus stubborn disease organisms form helical motile cells, and are now classified in a new genus, _Spiroplasma_.

Most plant pathogenic bacteria are capable of movement in water by means of the rotation of long threadlike flagella attached to the wall. The genus _Erwinia_ is characterized by random arrangement of flagella on the cell surface (peritrichous); in contrast the genus _Xanthomonas_ is characterized by a single flagellum attached at one end (polar).

It is not possible to separate plant pathogenic bacteria into species on the basis of cell morphology,

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or colony or staining characteristics. Therefore,
biochemical and physical tests used to differentiate
bacteria in general are also used in studies on plant
pathogens. Helpful techniques include serological
tests, DNA hybridization, sensitivity to phages, and
gel electrophoresis of proteins. However, one of the
most important of the tests for identification is the
demonstration of pathogenicity to a specific plant.

Viruses and Viroids

Viruses and viroids are the simplest of the
various causative agents of plant disease. The
essential element of each of these two pathogens is an
infective nucleic acid. The nucleic acid of viruses is
covered by an exterior shell (coat) of protein, but
that of viroids is not.

Approximately 400 plant viruses and about 10
viroids are known. The nucleic acid of most plant
viruses is a single-stranded RNA (mol. wt. 1.5-4
million); a number of isometric viruses have a double-
stranded RNA (mol. wt. 10-20 million). A few viruses
contain double-stranded DNA (mol. wt. 4-5 million), and
several containing single-stranded DNA (mol. wt. 0.8
million) have been reported. The nucleic acid of a
viroid is single-stranded RNA, but its molecular weight
(about 120,000) is much lower than that of viruses.

Viruses require living cells for their
replication. Some viruses, such as tobacco mosaic
virus (TMV) and cucumber mosaic virus (CMV), are found
in many plant species; others, such as wheat streak
mosaic virus, occur only in a few grasses. The
replication of a plant virus appears to proceed
according to the following general scheme:
Introduction of the virus to a plant through a wound;
release of the nucleic acid from the protein coat;
association of viral RNA (or messenger RNA of DNA

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viruses) with cellular ribosomes for its translation to
the proteins required for virus synthesis; replication
of the nucleic acid and production of coat protein; and
assembly of the nucleic acid and coat protein to form
complete virus particles. The replication of viroids
is not clearly understood at present. Cell-to-cell
spread of viruses usually occurs, and eventually the
virus spreads throughout the plant. In some plants,
the cells surrounding the initially infected cells die,
and the virus usually does not spread further.

     Plant diseases of particular interest include:
diseases caused by bacterial plant-associated pathogens
such as <u>Erwinia carotovora</u>, <u>Pseudomonas syringae</u>, and
<u>Xanthomonas campestris</u>, which have been isolated from
potato, tomato, and rapeseed and cotton plants,
respectively; diseases caused by fungal plant-
associated pathogens such as <u>Aspergillus flavus</u>,
<u>Fusarium oxysporum</u>, <u>Phytophthora infestans</u>, <u>Pythium
ultimum</u>, and <u>Whetzelinia sclerotiorum</u>, which have been
isolated from raw spanish peanuts and cotton, tomato,
cotton and cabbage plants, respectively. The fungus
<u>Verticillium albo-atrium</u> has been isolated from cotton
and tomato plants.

     Many other microbes are also known as plant-
associated pathogens, for example <u>Aspergillis
parasiticus</u>, <u>Alternaria brassicae</u>, <u>Alternaria raphani</u>,
<u>Leptospheria maculans</u>, <u>Candida poropsilosis</u> and <u>Phoma
lingam</u>, etc. A more detailed identification and
discussion of the species comprising plant-associated
pathogens is contained in Agrios, G.N., <u>Plant
Pathology</u>, 3rd ed., Academic Press, San Diego, 1988.

     Fairly recently, broad spectrum antimicrobial
peptides have been discovered which function as part of
the host defense system of some animals against
invading microorganisms: included are the families of
peptides known as defensins and magainins. The

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defensins and the magainins show promise in
health/veterinary-related applications because of their
wide range of biocidal activity against microbes
associated with common animal infections.

Mammalian defensins are low molecular weight
cationic peptides (usually 29-34 amino acids in length)
that have been found in human, rabbit, and guinea pig
polymorphonuclear leukocytes (PMN) and rabbit alveolar
macrophages. Other mammals, such as bovines, also
contain antimicrobial peptides which show different
protein sequences and different environmental
sensitivities. Defensins are disclosed generally in
U.S. Patent Nos. 4,543,252 and 4,659,692.

As used herein, the term defensin is taken to mean
cationic oligopeptides of 35 or less amino acids having
a sequence of the formula:

$$aa_1-aa_2-cys-aa_4-cys-arg-aa_7-aa_8-aa_9-cys-$$
$$aa_{11}-aa_{12}-aa_{13}-glu-arg-aa_{16}-aa_{17}-gly-aa_{19}-cys-$$
$$aa_{21}-aa_{22}-aa_{23}-gly-aa_{25}-aa_{26}-aa_{27}-aa_{28}-aa_{29}-cys-$$
$$cys-aa_{32}-w$$

wherein

w is as defined in U.S. Patent No. 4,659,692, and
   aa_1 is val or gly;
   aa_2 is val, ile, arg, ser or phe;
   aa_4 is ala, val or thr;
   aa_7 is arg, lys or gly;
   aa_9 is leu, leu-leu, phe or ser;
   aa_{11} is leu, pro, ser or gly;
   aa_{12} is pro, asn, lys, phe or ser;
   aa_{13} is arg, leu, ser or gly;
   aa_{16} is arg, phe or ala;
   aa_{17} is ala, ser or ile;
   aa_{19} is phe, tyr, asp or ser;
   aa_{21} is arg, lys or thr;
   aa_{22} is ile or val;
   aa_{23} is arg or asn;
   aa_{25} is arg, ala or val;
   aa_{26} is ile or arg;
   aa_{27} is his, val or phe;
   aa_{28} is pro, tyr or thr;
   aa_{29} is leu, arg or phe;
   aa_{32} is arg, ser or pro; and
   w is $(arg \text{ or } lys)_{0-1}$.

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Particularly preferred are defensins of the following amino acid sequences:

NP1   val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-
           pro-arg-glu-arg-arg-ala-gly-phe-cys-arg-ile-
           arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg

NP2   val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-
           pro-leu-glu-arg-arg-ala-gly-phe-cys-arg-ile-
           arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg

NP3a   gly-ile-cys-ala-cys-arg-arg-arg-phe-cys-pro-
           asn-ser-glu-arg-phe-ser-gly-tyr-cys-arg-val-
           asn-gly-ala-arg-tyr-val-arg-cys-cys-ser-arg-arg

NP3b   gly-arg-cys-val-cys-arg-lys-gln-leu-leu-cys-ser-
           tyr-arg-glu-arg-arg-ile-gly-asp-cys-lys-ile-
           arg-gly-val-arg-phe-pro-phe-cys-cys-pro-arg

NP4   val-ser-cys-thr-cys-arg-arg-phe-ser-cys-gly-
           phe-gly-glu-arg-ala-ser-gly-ser-cys-thr-val-
           asn-gly-val-arg-his-thr-leu-cys-cys-arg-arg

NP5   val-phe-cys-thr-cys-arg-gly-phe-leu-cys-gly-
           ser-gly-glu-arg-ala-ser-gly-ser-cys-thr-ile-
           asn-gly-val-arg-his-thr-leu-cys-cys-arg

In vivo, defensins are believed to exercise cytotoxic activity against a variety of microbes in conjunction with their release from granulocytes during phagocytosis. Defensin-cidal activity has been reported in vitro against various microorganisms, including some enveloped viruses (herpes simplex, types I and II), for example. Some microbes, such as nonenveloped viruses and Bordetella bronchiseptica, are reported to show resistance to defensins; others are inhibited at varying degrees of effectiveness.

Magainins, first reported in 1987, are a class of anti-microbial peptides isolated from Xenopus skin. The two first-characterized identified magainins (M1 and M2) each have 23 amino acids but differ by two amino acid substitutions. Both have been shown to inhibit the growth of several species of bacteria and fungi. These amphibian proteins are functionally and

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structurally distinct from defensins. Magainins are
disclosed generally in U.S. Patent No. 4,810,777.

As used herein, the term magainin is taken to mean
the class of oligopeptides of molecular weight of 2500
5   or less and having an amino acid sequence of the
formula:

aa_1-aa_2-aa_3-aa_4-aa_5-leu-his-ser-ala-aa_{10}-
lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
met-aa_{22}-aa_{23}

10   wherein

aa_1 is gly_{0-1};
aa_2 is ile_{0-1};
aa_3 is gly_{0-1};
aa_4 is lys_{0-1};
15   aa_5 is phe_{0-1};
aa_{10} is lys or gly;
aa_{22} is asn or lys; and
aa_{23} is ser_{0-1}.

Particularly preferred are magainins of the
20   following amino acid sequences:

M1   gly-ile-gly-lys-phe-leu-his-ser-ala-gly-
          lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
          met-lys-ser

M2   gly-ile-gly-lys-phe-leu-his-ser-ala-lys-
25   lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
          met-asn-ser

M2(a)   ile-gly-lys-phe-leu-his-ser-ala-lys-
          lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
          met-asn-ser

30   M2(b)   gly-lys-phe-leu-his-ser-ala-lys-
          lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
          met-asn-ser

M2(c)   lys-phe-leu-his-ser-ala-lys-
          lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
35   met-asn-ser

M2(d)   phe-leu-his-ser-ala-lys-
          lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
          met-asn-ser

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M2(e)   leu-his-ser-ala-lys-
             lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
             met-asn-ser

M3   gly-ile-gly-lys-phe-leu-his-ser-ala-lys-
             lys-phe-gly-lys-ala-phe-val-gly-glu-ile-
             met-asn

    According to this invention, it has been found
that these animal-derived AMP's show activity against a
wide range of plant pathogens as well.

    By animal-derived anti-microbial peptides (AMP) is
meant any protein purified from animal sources,
synthetically constructed, or combination thereof,
functionally based upon the defensin or magainin
properties.

    The nomenclature used to describe AMP compounds of
the present invention follows the conventional practice
wherein the amino group is presented to the left and
the carboxy group to the right of each amino acid
residue. In the formulae representing embodiments of
the present invention, the Amino- and Carboxy-terminal
groups, when not specifically shown, will be understood
to be in the form they would assume at physiologic pH
values, unless otherwise specified.

    In the amino acid sequence formulae presented
herein each residue is specified by the conventional
practice of using three letters from the trivial name
of the amino acid and wherein the L form of any amino
acid having an optical isomer is intended unless
otherwise expressly indicated.

    In accordance with this invention, it is possible
to limit the growth of such plant-associated pathogens
by contact with an animal-derived antimicrobial
peptide. Contact must occur under conditions in which
the antimicrobial peptides retain biological activity
(i.e., within particular parameters of pH, temperature,
etc.). Depending upon the circumstances, slowing of

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the growth of the plant pathogen may be sufficient, and
in some circumstances, a slowed growth may be
preferred. In other cases, complete inhibition of the
pathogen's growth may be desired. Depending upon the
5   desired effect and the specific plant pathogens of
interest, different animal-derived AMP's may be useful
and the concentration of such AMP modified. The means
of contact is not critical to this invention and may
include any means of administering, targeting,
10   delivering, applying, directing, or facilitating a
biochemical reaction between the AMP and the target
microbial plant pathogen. The animal-derived AMP may
be used in any form which retains antimicrobial
activity. Examples of some acceptable modes are
15   provided in United States patents 4,543,252, 4,659,692
and 4,705,777. One skilled in the art will find other
variations permissible as well.

In accordance with one aspect of the subject
Very little is known about the toxicity of the
animal-derived AMP's to the cells of higher organisms.
20   By this invention, the effect of these animal-derived
AMP's on plant tissues becomes of interest. With
respect to plant tissues, tests indicate that effects
of defensins and magainins on plant cell protoplasts
may be dose dependent. No cell lysis is observed at
25   concentrations of less than 0.25mg/ml of peptide for
the majority of peptides tested. Depending upon the
agent, lysis of tobacco cell protoplasts was noted in
the range of 2.5 to 0.25 mg/ml.

In accordance with one aspect of the subject
30   invention, DNA constructs are provided which include
the structural gene sequence for expression of selected
AMP's, which constructs may be introduced into a
variety of plant hosts in a variety of ways and, for
example, may be present as an episomal element or
35   integrated into the host chromosome.

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The structural genes for selected AMPs can be obtained for any AMP with a known amino acid sequence and/or generation of a genomic DNA library from the source for a selected AMP. The DNA sequence containing the AMP structural gene of interest may then be joined to a wide variety of other DNA sequences for introduction into an appropriate host cell. The companion sequence will depend largely upon the nature of the host, the manner of introduction of the DNA sequence into the host, and whether episomal maintenance or integration is desired. For example, the structural gene as part of a construct may be introduced into a plant cell nucleus by micropipet injection for integration by recombination into the host genome.

Alternatively, temperate viruses may be employed into which the structural gene may be introduced for introduction into a plant host. Where the structural gene has been obtained from a source having regulatory signals which are not recognized by the plant host, it may be necessary to introduce the appropriate regulatory signals for expression.

Where a virus or plasmid, e.g., tumor inducing plasmid, is employed and has been mapped, a restriction site can be chosen which is downstream from a promoter into which the structural gene may be inserted at the appropriate distance from the promoter. Where the DNA sequences do not provide an appropriate restriction site, one can digest back portions of the DNA sequence for various times with an exonuclease, such as Bal31 and insert a synthetic restriction endonuclease site. Methods for introducing viruses and plasmids into plants are described in the literature (e.g., Matzke and Schulton, J. Mol. App. Genetics 1:39-49 (1981)).

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Of particular interest is the use of a tumor-
inducing plasmid, e.g., Ti or Ri, where the AMP
structural gene may be integrated into plant cell
chromosomes. By employing the Ti-DNA right and left
5   borders, where the borders flank an insert comprising
the AMP structural gene under transcriptional and
translational regulatory signals recognized by the
plant host, the construct may be integrated into the
plant genome and provided for expression of the AMP in
10   the plant cell at various stages of differentiation.
As transcriptional and translational regulatory
regions, conveniently a signal peptide from a barley
thionin may be employed which allow for transport of
the AMP out of the plant cell. Promoters and/or
15   terminators may be employed, including promoters that
would provide for inducible expression or regulated
expression and a plant host. Promoter regions which
may be used from the Ti plasmid include the octopine
synthase promoter, nopaline synthase promoter, agropine
20   synthase promoters, or the like. Other promoters
include viral promoters such as CaMV region VI promoter
or full length promoter, the promoters associated with
the ribulose-1,5-bisphosphate carboxylate genes, e.g.,
the small sub-unit genes associated with phaseolin,
25   protein storage, cellulose formation, or the like.
The various sequences may be joined together in
conventional ways. The promoter region may be
identified by the region being 5' from the structural
gene, for example, the opine gene, and by restriction
30   mapping and sequencing may be selected and isolated.
Similarly, the terminator region may be isolated as the
region 3' from the structural gene. The sequences may
be cloned and joined in the proper orientation to
provide for constitutive expression of the structural
35   gene in a plant host. By modifying crop plant cells by
introduction of a functional structural gene expressing

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a selected AMP, one can provide a wide variety of crops which have enhanced resistance to a broad spectrum of plant-associated pathogens, without requiring continual crop treatment and while leaving the crop relatively unaffected. In this manner, substantial economies can be achieved in labor and materials while minimizing the detrimental effects of the plant associated pathogens.

The AMP structural gene may be introduced into a wide variety of plants, both monocotyledon and dicotyledon, including maize, wheat, soy bean, tobacco, cotton, tomatoes, potatoes, Brassica species, rice, peanuts, petunia, sunflower, sugar beet, turf grass, etc. The gene may be present in cells or plant parts including callus, roots, tubers, propagules, plantlets, seed, seedlings, pollen, or the like. The AMP structural gene will manifest its activity by limiting the growth of plant associated pathogens which contact the genetically modified plant or cells thereof.

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Experimental

In the experimental disclosure which follows, all weights are given in grams (g), milligrams (mg) or micrograms (μg), all concentrations are given as percent by weight (%), molar (M), millimolar (mM) or micromolar (μM), and all volumes are given in liters (l) or milliliters (ml) unless otherwise indicated.

In order to demonstrate the use of AMP compounds in accordance with the present invention, the following examples demonstrate the inhibitory effects of AMPs applied to Plant-Associated Pathogens.

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Example 1:   Defensin AMP Compounds

    Defensin AMP compounds may be obtained as follows:
AMP compounds known as Defensins, including rabbit
neutrophil proteins ("NP") NP1, NP3A, NP5 and human
5   neutrophil proteins ("HNP") HNP1, can be prepared as
described in U.S. Patent Nos. 4,543,252, 4,659,692 and
4,705,777.

    One mg of each pure (verified by NMR) lyophilized
defensin protein preparation to be employed is
10   resuspended in 100µl of 0.01% (v/v) filter sterilized
acetic acid, respectively.

    It has been found that commercially available agar
may contain contaminants which inhibit the action of
defensins. For testing of defensins, a medium with low
15   contaminants is needed. A representative example of
such a medium includes Czapek Dox Broth (Difco) plus
1.5% purified agarose (Sea Plaque® agarose from FMC).

Example 2:   Magainin AMP Compounds

    Magainin AMP compounds may be obtained as follows:
20   Magainins can be prepared as described in U.S. Patent
No. 4,810,777. Magainins M1 and M2 can also be
obtained commercially (for example, United States
Biochem Corp., OH: catalog nos. 18649 and 18652,
respectively).

25   500µg of each lyophilized Magainin protein
preparation obtained as described is resuspended in
50µg sterile distilled H₂O, respectively.

Example 3:   Fungal Plant-Associated Pathogens

    The inhibition of fungal PAP growth by application
30   of AMPs is demonstrated employing defensins and
magainins in AMP-filled plug wells or AMP saturated
paper disks (BBL/Becton Dickenson Co., MD: catalog
#31039) on petri-dishes containing a medium such as
Czapek Dox Broth (Difco) plus 1.5% purified agarose

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(Sea Plaque® agarose from FMC). Either method appears
to provide satisfactory results.

In the plug-well method, wells are punched in a
concentric pattern into Czapek Dox medium-filled petri-
dishes (35x10mm) using a Pasteur pipet. The plates are
then inoculated in the center with a 2mm fungal plug
and left until fungal growth is approximately halfway
between the initial plug and the wells. At this point,
$2.5-20 \mu g$ of the selected AMPs are added to the wells in
a small volume ($2-5 \mu l$). The plates are then sealed
with Parafilm® and fungal growth is monitored daily for
signs of inhibition.

In a paper disk method, the plate is inoculated as
above and then sterile pie-shaped paper disks are
placed outside of the region of fungal growth. The AMP
to be tested is then pipetted onto the paper disk. All
other procedures remain the same.

Fungal PAP strains employed for testing are
available from the ATCC: Aspergillus flavus ATCC
15547; Aspergillus parasiticus ATCC 26691; Fusarium
oxysporum f. sp. vasinfectum ATCC 16611; Pythium
ultimum ATCC 56081; and Verticillium albo-atrium ATCC
18698 (isolated from V-81, tomato) and ATCC 18699
(isolated from V-43, cotton).

Fusarium oxysporum, Pythium ultimum, and both
strains of Verticillium tested show significant
inhibition from all magainins and defensins tested.
The fungal growth was markedly limited in areas
adjacent to the animal-derived AMP proteins to varying
degrees. A summary of selected results of such
inhibition is found in Table 1.

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Table 1

Fungal Growth Inhibition Activity of AMP Compounds

Pathogen	AMP Compound						
	NP1	NP3A	NP5	HNP1	M1	M2	C
Fusarium oxysporum	+++	+++	+	+++	+	+	-
Pythium ultimum	+++	+++	+	+++	+	+	-
Verticillium albo-atrium V-81 (18698)	+++	+++	+	+++	+++	+	-
Verticillium albo-atrium V-43 (18699)	+++	++	+	+++	+	-	-

"C" is control; "+" indicates some inhibition activity; "+++" indicates relatively strong inhibition activity; "-" indicates a lack of apparent inhibition activity.

A line of precipate was noted to form in the zone of inhibition of Verticillium V43 and V81 strains with NP1, NP3A, and HNP1. The effect of the animal-derived AMP's on Aspergillus was inconclusive.

5   ## Example 4: Bacterial Plant-Associated Pathogens

Bacterial PAPs to be tested are grown in 10ml of 2YT liquid medium (16g tryptone, 10g yeast extract, 5g NaCl and 1000ml DDH$_2$O, pH adjusted to 7.0 with 1M NaOH) until mid-log phase (OD$_{600}$=approximately 0.5), washed
10   two times with 10ml of 10mM potassium phosphate buffer, pH 7.4 and centrifuged at 2000xg for 10 minutes.

The bacteria are resuspended in buffer and the OD measured and adjusted to 0.5 (0.5 OD$_{600}$=4x10^8 cells/ml). The cells are then diluted to approximately 10^5 cells/ml
15   in either 10mM phosphate buffer alone (control) or buffer plus AMP at final concentrations from 1-10μg/ml. The treated cells are then incubated at 37°C for 1 hour. The incubation is terminated by further dilution (1/100 and 1/1000) and 100μl of the diluted cells are
20   plated on semi-solid 2YT medium (2YT media plus 1.5% agar) in duplicate. The plates are then incubated overnight at 37°C and colony counts are determined.

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Inhibition of bacteria is measured as the
reduction in the number of colonies when compared to
controls. Bacteria known to be inhibited by the
magainin and/or defensin proteins, such as _E. coli_, _S._
5   _aureus_, or _P. aeurginosa_, are tested as controls in
these examples.

Bacterial PAP strains employed for testing herein
are available from the ATCC: _Erwinia carotovora subsp._
carotovora ATCC 25272; _Pseudomonas syringae pv._ tomato
10   ATCC 10862 (U.S. Patent 3,066,080); and _Xanthamonas_
campestris pumalvacearum XM 13 cotton ATCC9924.

At AMP concentrations of 10µg/ml, varying degrees
of inhibition were seen. Complete inhibition of growth
was demonstrated in some instances. A summary of
15   selected results in found in Table 2.

Table 2

Bacterial Growth Inhibition Activity of AMP Compounds
Percent Inhibition at 10µg/ml Peptide Concentration

Pathogen	AMP Compound						
	NP1	NP3A	NP5	HNP1	M1	M2	C
Erwinia carotovora	100	69	62	59	62	99	0
Pseudomonas syringae	100	100	70	0	52	100	0
Xanthamonas campestris	100	100	34	14	92	99	0

"C" is control.

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Some bacterial growth inhibition was also observed
with AMPs at peptide concentrations of 1μg/ml: NP1
demonstrated strong inhibition of Erwinia (97%) and a
slightly lesser inhibition of Xanthamonas (88%).

5   Relatively strong inhibitory effects were demonstrated
by NP3A for the same strains, in the range of 60-75%
inhibition. Some inhibitory effects are seen in
Pseudomonas as well: NP1 (22% inhibition), HNP1 (33%
inhibition) and M1 (39% inhibition).

10   In order to demonstrate additional embodiments of
the present invention, the following examples
illustrate the construction of DNA sequences for
expression and transport of AMPs in plant tissue and
the transformation of plant tissue therewith.

15 Example 5: Synthesis of the Thionin Signal Peptide/NP1
DNA Sequence

The DNA sequence for expression and transport of
the defensin NP1 (rabbit) is assembled from synthetic
oligonucleotides using the Klenow fragment of DNA

20 polymerase and the polymerase chain reaction (PCR)
technique, as described in U.S. Patent Nos. 4,683,195
and 4,683,202.

The DNA sequence for expression and transport of
NP1 consists of six non-specific base pairs followed by

25 a BamHI site, then the DNA sequence encoding the
putative signal peptide from a barley leaf-specific
thionin (to allow for transport out of the cell) fused
in-frame to the mature rabbit NP1 coding region, an
Asp718 site, and six more non-specific base pairs. The

30 BamHI and Asp718 sites allow the gene to be cloned into
the double 35S expression cassette pCGN1431 (described
below); the non-specific bases on the ends are employed
to provide double-stranded helix structure on both
sides of the restriction sites, a feature which most

35 restriction enzymes require for activity. The codons

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are selected in the design of this DNA sequence based on codon preferences for dicotyledonous plants (Murray et al., Nucl. Acids Res. 17:477-498 (1989)) to allow maximal translation rate in the target plants of the present example (Brassica sp.). Codon selection for other target plant species will be made as a matter of ordinary skill.

The DNA sequence which encodes the putative signal peptide of barley leaf thionin is 127bp in length. Two overlapping oligonucleotides were designed which, when annealed and extended with Klenow fragment, comprise the entire sequence: "SIG-T" is 73 "sense" strand bases, consisting of bases of 1-73 of the DNA sequence encoding the thionin signal peptide (as described by Gausing, Planta 171:241-246 (1987)):

"SIG-T":

5'-CATATCTCATTCTACTTGAGAAATTAAGGCCAACC

AGCCAACCATGGCAACCAACAAGTCTATTAAGTCCGTG-3'

"SIG-B" is 74 "anti-sense" strand bases, consisting of bases 127-54 of the DNA sequence encoding the signal peptide (Gausing, (1987) supra).

"SIG-B":

5'-GGCTTCAACTTGGACCTGCTCAAGAACCAAACCCAA

TATAAGAACACAAATAACCACGGACTTAATAGACTTGT-3'

Bases 54-73 of "SIG-T" and "SIG-B" overlap as complementary strands, and when allowed to anneal they can be extended with Klenow fragment to yield a full length double-stranded DNA fragment of 127bp.

In practice, only a relatively small fraction of the polymerase reaction products will comprise the full 127 base pairs, since the synthesis of long oligomers (>40 bases) is not highly efficient. By employing the

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PCR as described below, the full length molecules can be selectively amplified, reducing the quantity of short fragments to an insignificant level.

The NP1 coding region is 102bp in length. It was constructed in a manner similar to the signal peptide DNA sequence, with "NP1-T" being "sense" bases 1-60, and "NP1-B" being "antisense" bases 102-41, based on the NP1 DNA sequence (as reported by Selsted and Harwig, Infect. Immun. 55(9):2281-2286 (1987)):

"NP1-T":

5'-GTTGTTTGCGCTTGTAGGAGAGCTTTGTGTCTTCCAAG AGAAAGGAGAGCTGGATTTTGC-3'

"NP1-B":

5'-TTATCTCCTACAGCACAATGGATGAATTCTTCCTCTA ATCCTGCAAAATCCAGCTCTCCTTT-3'

These oligonucleotides were annealed and repaired with Klenow fragment as described above.

Four smaller oligonucleotides were synthesized in order to allow fusion of the signal sequence and the NP1 sequence, facilitate addition of the restriction sites, and allow for amplification of full-length fragments from the above polymerase reactions:

"SIG-L" is a 33 base oligonucleotide consisting of six nonspecific bases, six bases comprising the BamHI site, and the first 21 "sense" bases (1-21) of the signal peptide DNA sequence:

5'-CGAGTTGGATCCCATATCTCATTCTACTTGAGA-3'

"NP1-R" is a 33 base oligonucleotide consisting of six nonspecific bases, six bases comprising the Asp718 site, and the last 21 "anti-sense" bases (102-82) of the NP1 sequence:

5'-TGAGTTGGTACCTTATCTCCTACAGCACAATGG-3'

"F-SN-T" is a 42 base oligonucleotide consisting
of the last 21 "sense" bases of the signal peptide
(107-127) and the first 21 "sense" bases of the
NP1 sequence (1-21) :

5'-GAGCAGGTCCAAGTTGAAGCCGTTGTTTGCGCTTGTAGGAGA-3'

"F-SN-B" is a 42 base pair oligonucleotide
complementary to "F-SN-T", consisting of
"anti-sense" bases 21-1 of NP1 and "anti-sense"
bases 127-107 of the signal peptide DNA sequence:

5 '-TCTCCTACAAGCGCAAACAACGGCTTCAACTTGGACCTGCTC-3'

When "SIG-L" and "F-SN-B" are used as primers in
the PCR with the repaired signal peptide DNA fragment
as template, the resulting product is a 160bp fragment
with the six non-specific base pairs and BamHI site,
the signal peptide DNA sequence, and 21 base pairs of
the NP1 DNA sequence. The PCR is performed using a DNA
Thermal Cycler machine (Perkin Elmer Cetus; Emeryville,
CA) in accordance with the instructions of the
manufacturer.

When "NP1-R" and "F-SN-T" are used as primers in
the PCR with the repaired NP1 DNA fragment as template,
the resulting product is a 135bp fragment with 21 base
pairs of the signal peptide DNA sequence, the NP1 DNA
sequence, the Asp718 site, and six nonspecific base
pairs. Each of these reactions selectively amplifies
only the full-length or near-full-length fragments from
the initial repair reactions, permitting the use of
crude oligonucleotides in all steps, and resulting in a
great savings of time and manpower.

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These two double-stranded products share a 42bp
overlap which defines the fusion junction for the
thionin signal sequence and the NP1 sequence. Upon
strand melting (such as occurs during the PCR), a
portion of each type will reanneal with an overlapping
strand of the other, forming a complex which can repair
into the full-length DNA sequence. When the two
fragments are mixed in a 1:1 ratio, and amplified in
the PCR using "SIG-L" and "NP1-R" as primers, the
resulting product is the full-length sequence with the
appropriate restriction sites attached.

The actual assembly of the DNA signal/NP1 sequence
was carried out in several steps. One nmol each of
"NP1-B" and "NP1-T" (for the NP1 region; "SIG-T" and
"SIG-B" were used for the signal region) were combined
with 1μl 100XTE (1XTE: 10mM Tris-HCl, 1mM EDTA) in a
total volume of 100μl. This mixture is placed in a DNA
Thermal Cycler (Perkin Elmer Cetus; Emeryville, CA) and
annealed for 30 minutes in a cooling gradient from 80°C
to 37°C followed by 15 minutes at 37°C. Ten μl of this
reaction was treated with Klenow fragment in a 50μl
reaction volume to repair the strands. One μl of the
Klenow fragment reaction was used in a PCR reaction
with "F-SN-T" and "NP1-R" ("F-SN-B" and "SIG-L") as
primers to add the fusion region. The PCR conditions
were as follows: 94°C for 1 min., 55°C for 2 min., 72°C
for 3 min. for a total of 25 cycles.

The products from each PCR reaction (resulting in
the NP1 region and the signal region) are then used as
template in another PCR reaction: Samples from each
reaction are combined in a one-to-one ratio and
subjected to the PCR using primers "SIG-L" and "NP1-R"
at 94°C for 1 min., 50°C for 2 min., 72°C for 3 min.
for a total of 25 cycles. Products of the PCR were
then digested with Asp718 and BamHI and cloned into
pCGN1431 (see below).

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Following digestion with BamHI and Asp718, the
thionin signal/NP1 DNA sequence was cloned into BamHI-
Asp718 digested pCGN1431. The sequence of the thionin
signal/NP1 fusion was confirmed by dideoxy chain
termination DNA sequencing (Sanger, et al., Proc.
Natl. Acad. Sci. USA 74:5463-5467 (1977)). The
resulting construct has been designated pCGN3700.

The expression cassette is then cloned into a
binary vector, such as pCGN1557 (described below) and
the resulting construct is introduced into
Agrobacterium tumefaciens by transformation. The
transformed A. tumefaciens is then used to generate
transformed plants, as described in Example 7.

Construction of pCGN1431

Plasmid pCGN1431 is described in co-pending
application U.S. Serial No. 07/225,332, which is
incorporated herein by this reference.

Plasmid pCGN1431 contains a double Cauliflower
Mosaic Virus (CaMV) promoter and the tml-3' region with
multiple cloning sites between them. This
promoter/terminator cassette is contained in a
pUC-derived vector which contains a chloramphenicol
resistance gene. The cassette is bordered by multiple
restriction sites for easy removal. The plasmid is
derived by a series of steps (see below) from an
initial double 35S plasmid, pCGN2113, which itself is
derived from pCGN164 and pCGN638. The plasmid pCGN2113
was deposited with the ATCC on March 22, 1989, under
accession number 40587.

The SalI-EcoRI fragment of pCGN2113 (see below),
which contains the entire promoter-polylinker-3'
cassette, is removed by SalI-EcoRI digestion and cloned
into SalI-EcoRI digested pCGN565 to create pCGN2120;
pCGN565 is cloning vector based on pUC12-Cm (K.
Buckley, "Regulation and expression of the φX174 lysis
gene", Ph.D. Thesis, University of California, San

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Diego, 1985), but containing the polylinker from pUC18
(Norrander et al., Gene 26:101-106 (1983)). Plasmid
pCGN2120 is digested to completion with PstI and
religated. A clone is selected which has deleted only
the 858bp PstI-PstI fragment (bp 9207-10065, Barker et
al., Plant Mol. Bio. 2:335-350 (1983)) from the tml-3'
region to create pCGN1431.

Construction of pCGN2113

Plasmid pCGN164 (see below) is digested with EcoRV
and BamHI to release a EcoRV-BamHI fragment which
contains a portion of the 35S promoter (bp 7340-7433,
Gardner et al., Nucl. Acids Res. 9:2871-2888 (1981)).
Plasmid pCGN638 (see below) is digested with HindIII
and EcoRV to release a HindIII-EcoRV fragment
containing a different portion of the 35S promoter
(bp 6493-7340). These two fragments are ligated into
pCGN986 (see below) which has been digested with
HindIII and BamHI to remove the HindIII-BamHI fragment
containing the 35S promoter. This ligation produces
pCGN639, which contains the backbone and tml-3' region
from pCGN986 and the two 35S promoter fragments from
pCGN164 and pCGN638. Plasmid pCGN638 is digested with
EcoRV and DdeI to release a fragment of the 35S
promoter (bp 7070-7340). The fragment is treated with
Klenow fragment to create blunt ends, and is ligated
into the EcoRV site of pCGN639 to produce pCGN2113,
having the fragment in the proper orientation.

Construction of pCGN164

The AluI fragment of CaMV (bp 7144-7735) (Gardner
et al., (1981) supra) is obtained by digestion with
AluI and cloned into the HincII site of M13mp7 (Vieira
and Messing, Gene 19:259-268 (1982)) to create C614.
An EcoRI digest of C614 produces the EcoRI fragment
from C614 containing the 35S promoter which is cloned
into the EcoRI site of pUC8 (Vieira and Messing, (1982)
supra) to produce pCGN146. To trim the promoter

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region, the BglII site (bp 7670) is treated with BglII
and Bal31 and subsequently a BglII linker is attached
to the Bal31 treated DNA to produce pCGN147. Plasmid
pCGN147 is digested with EcoRI and HphI and the
resultant EcoRI-HphI fragment containing the 35S
promoter is ligated into EcoRI-SmaI digested M13mp8
(Vieira and Messing, (1982) supra) to create pCGN164.

Construction of pCGN638

Digestion of CaMV10 (Gardner et al., (1981) supra)
with BglII produces a BglII fragment containing a 35S
promoter region (bp 6493-7670) which is ligated into
the BamHI site of pUC19 (Yanisch-Perron et al., Gene
53:103-119 (1985)) to create pCGN638.

Construction of pCGN1557

Plasmid pCGN1557 (McBride and Summerfelt, Plant
Molecular Biology 14(2):269-276 (1990)) is a binary
plant transformation vector containing the left and
right T-DNA borders of Agrobacterium tumefaciens
octopine Ti-plasmid pTiA6 (Currier and Nester, J. Bact.
126:157-165 (1976)), the gentamicin resistance gene of
pPh1JI (Hirsch and Beringer, Plasmid 9:2871-2890
(1984)), an Agrobacterium rhizogenes Ri plasmid origin
of replication from pLJbB11 (Jouanin et al., Mol. Gen.
Genet. 201:370-374 (1985)), a 35S promoter-Kan^r-tml-3'
region capable of conferring kanamycin resistance to
transformed plants, a ColE1 origin of replication from
pBR322 (Bolivar et al., Gene 2:95-133 (1977)), and a
lacZ' screenable marker gene from pUC18 (Norrander et
al., Gene 26:101-106 (1983)).

There are three major intermediate constructs used
to generate pCGN1557:

pCGN1532 (see below) contains the pCGN1557
backbone, the pRi origin of replication, and the ColE1
origin of replication;

pCGN1546 (see below) contains the CaMV 35S 5'-
Kan^r-tml-3' plant selectable marker region; and

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pCGN1541b (see below) contains the right and left T-DNA borders of the A. tumefaciens octopine Ti-plasmid and the lacZ' region from pUC19.

To construct pCGN1557 from the above plasmids, pCGN1546 is digested with XhoI, and the fragment containing the CaMV 35S 5'-Kan^r-tml-3' region is cloned into the XhoI site of pCGN1541b to give the plasmid pCGN1553, which contains T-DNA/left border/CaMV 35S 5'-Kan^r-tml-3'/lacZ'/T-DNA left border. Plasmid pCGN1553 is digested with BglII, and the fragment containing the T-DNA/left border/CaMV 35S 5'-Kan^r-tml-3'/lacZ'/T-DNA left border region is ligated into BamHI digested pCGN1532 to give the complete binary vector, pCGN1557.

Construction of pCGN1532

The 3.5kb EcoRI-PstI fragment containing the gentamicin resistance gene is removed from pPh1JI (Hirsch and Beringer, (1984) supra) by EcoRI-PstI digestion and cloned into EcoRI-PstI digested pUC9 (Vieira and Messing, (1982) supra) to generate pCGN549. HindIII-PstI digestion of pCGN549 yields a 3.1kb fragment bearing the gentamicin resistance gene, which is made blunt ended by Klenow fragment and cloned into PvuII digested pBR322 (Bolivar et al., (1977) supra) to create pBR322Cm. Plasmid pBR322Cm is digested with DraI and SphI, treated with Klenow fragment to create blunt ends, and the 2.8kb fragment cloned into the Ri origin-containing plasmid pLJbB11 (Jouanin et al., (1985) supra) which has been digested with ApaI and made blunt-ended with Klenow fragment, creating pLHbB11Gm. The extra ColE1 origin and the kanamycin resistance gene (Kan^r) are deleted from pLHbB11Gm by digestion with BamHI followed by self closure to create pGmB11. The HindIII site of pGmB11 is deleted by HindIII digestion followed by treatment with Klenow fragment and self closure, creating pGmB11-H. The PstI site of pGmB11-H is deleted by PstI digestion followed

by treatment with Klenow fragment and self closure,
creating pCGN1532.

Construction of pCGN1546

The 35S promoter-tml-3' expression cassette,
pCGN986, contains a CaMV 35S promoter and a T-DNA tml-
3'-region with multiple restriction sites between them.
Plasmid pCGN986 is derived from another cassette,
pCGN206, containing a CaMV 35S promoter and a different
3' region, the CaMV region VI 3'-end. The CaMV 35S
promoter is cloned as an AluI fragment (bp 7144-7734)
(Gardner et al., (1981) supra) into the HincII site of
M13mp7 (Messing et al., Nucl. Acids Res. 9:309-321
(1981)) to create C614. An EcoRI digest of C614
produced the EcoRI fragment from C614 containing the
35S promoter which is cloned into the EcoRI site of
pUC8 (Vieira and Messing, (1982) supra) to produce
pCGN147.

Plasmid pCGN148a containing a promoter region,
selectable marker (Kan^r with 2 ATG's) and 3' region, is
prepared by digesting pCGN528 with BglII and inserting
the BamHI-BglII promoter fragment from pCGN147. This
fragment is cloned into the BglII site of pCGN528 so
that the BglII site is proximal to the Kan^r gene of
pCGN528.

The shuttle vector used for this construct
pCGN528, is made as follows: pCGN525 is made by
digesting a plasmid containing Tn5 which harbors a Kan^r
gene (Jorgenson et al., Mol. Gen. Genet. 177:65 (1979))
with HindIII and BamHI and inserting the HindIII-BamHI
fragment containing the Kan^r into the HindIII-BamHI site
in the tetracycline resistance (Tet^r) gene of pACYC184
(Chang and Cohen, J. Bacteriol. 134:1141-1156 (1978)).
Plasmid pCGN526 is made by inserting the BamHI fragment
19 of pTiA6 (Thomashow et al., Cell 19:729-739 (1980)),
modified with XhoI linkers inserted into the SmaI site,
into the BamHI site of pCGN525. Plasmid pCGN528 is

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obtained by deleting the small XhoI fragment from
pCGN526 by digesting with XhoI and religating.

Plasmid pCGN149a is made by cloning the BamHI-
Kan^r fragment from pMB9KanXXI into the BamHI site of
pCGN148a. Plasmid pMB9KanXXI is a pUC4K variant
(Vieira and Messing, (1982) supra) which has the XhoI
site missing, but contains a functional Kan^r gene from
Tn903 to allow for efficient selection in
Agrobacterium.

Plasmid pCGN149a is digested with HindIII and
BamHI and ligated with HindIII and BamHI digested pUC8
to remove the Tn903 Kan^r marker and produce pCGN169.
Plasmids pCGN565 (a cloning vector based on pUC12-cm
(K. Buckley, (1985) supra) but containing pUC18
linkers) and pCGN169 are both digested with HindIII and
PstI and ligated to form pCGN203, a plasmid containing
the CaMV 35S promoter and part of the 5'-end of the Tn5
Kan^r gene (up to the PstI site, Jorgenson et al., (1979)
supra). A 3'-regulatory region is added to pCGN203
from pCGN204 (an EcoRI fragment of CaMV (bp 408-6105)
containing the region VI 3' cloned into pUC18
(Norrander et al. (1983) supra)) by digestion with
HindIII and PstI and ligation. The resulting cassette,
pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml-3'-sequences are subcloned
from the BamHI fragment 19 T-DNA fragment (Thomashow et
al., (1980) supra) as a BamHI-EcoRI fragment (bp 9062-
12,823, numbering as in Barker et al., (1983) supra)
and combined with the pACYC184 (Chang and Cohen,
(1978), supra) origin of replication as an EcoRI-
HindIII fragment and a gentamicin resistance marker
(from plasmid pLB41, obtained from D. Figurski,
University of California, San Diego) as a BamHI-HindIII
fragment to produce pCGN417.

The unique SmaI site of pCGN417 (nucleotide 11,207
of the Bam19 fragment) is changed to a SacI site using

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linkers and the BamHI-SacI fragment is subcloned into
pCGN565 to give pCGN971. The BamHI site of pCGN971 is
changed to an EcoRI site using linkers. The resulting
EcoRI-SacI fragment containing the tml-3' regulatory
sequences is joined to pCGN206 by digestion with EcoRI
and SacI to give pCGN975. The small part of the Tn5
Kan^r gene is deleted from the 3'-end of the CaMV 35S
promoter by digestion with SalI and BglII, blunting the
ends and ligation with SalI linkers. The final
expression cassette pCGN986 contains the CaMV 35S
promoter followed by two SalI sites, an XbaI site,
BamHI, SmaI, KpnI and the tml-3' region (bp 11207-9023
of the T-DNA).

The CaMV 35S promoter-tml-3' expression cassette,
pCGN986 is digested with HindIII. The ends are filled
in with Klenow fragment and XhoI linkers are added.
The resulting plasmid is called pCGN986X. The BamHI-
SacI fragment of pBRX25 (see below) containing the
nitrilase gene is inserted into BamHI-SacI digested
pCGN986X yielding pBRX66.

Construction of pBRX25 is described in U.S. Patent
No. 4,810,648, which is hereby incorporated by
reference. Briefly, the method is as follows: The
nucleotide sequence of a 1212bp PstI-HincII DNA segment
encoding the bromoxynil-specific nitrilase contains
65bp of 5'-untranslated nucleotides. To facilitate
removal of a portion of these excess nucleotides,
plasmid pBRX9 is digested with PstI, and treated with
nuclease Bal31. BamHI linkers are added to the
resulting ends. BamHI-HincII fragments containing a
functional bromoxynil gene are cloned into the BamHI-
SmaI sites of pCGN565. The resulting plasmid, pBRX25,
contains only 11bp of 5'-untranslated bacterial
sequence.

Plasmid pBRX66 is digested with PstI and EcoRI,
blunt ends generated by treatment with Klenow fragment,

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and XhoI linkers added. The resulting plasmid pBRX68
now has a tml-3' region that is approximately 1.1kb.
Plasmid pBRX68 is digested with SalI and SacI, blunt
ends generated by treatment with Klenow fragment and
EcoRI linkers added. The resulting plasmid, pCGN986XE
is a 35S promoter-tml-3' expression cassette lacking
the nitrilase gene.

The Tn5 Kan^r gene is then inserted into pCGN986XE.
The 1.0kb EcoRI fragment of pCGN1536 (see below) is
ligated into pCGN986XE digested with EcoRI. A clone
with Kan^r in the correct orientation for transcription
and translation is chosen and called pCGN1537b. The
35S promoter Kan^R-tml-3' region is then transferred to a
chloramphenicol resistant (Cam^r) plasmid backbone.
Plasmid pCGN786, (a pUC-CAM based vector with the
synthetic oligonucleotide
5' GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3'
containing the cloning sites EcoRI, SalI, BglII, PstI,
XhoI, BamHI, and HindIII inserted into pCGN566; pCGN566
contains the EcoRI-HindIII linker of pUC18 inserted
into the EcoRI-HindIII sites of pUC13-cm (K. Buckley,
(1985) supra)) is digested with XhoI and the XhoI
fragment of pCGN1537b containing the 35S promoter Kan^r-
tml-3' region is ligated in. The resulting clone is
termed pCGN1546.

Construction of pCGN1536

The 5.4kb EcoRI fragment is removed from pVK232
(Knauf and Nester, Plasmid 8:45 (1982)) by EcoRI
digestion and cloned into EcoRI digested pACYC184
(Chang and Cohen, (1978) supra) to create pCGN14. The
1434bp ClaI-SphI fragment of pCGN14, containing the
mas-5' region (bp 20128-21562 according to numbering of
Barker et al., (1983) supra) is cloned into AccI-SphI
digested pUC19 (Yanisch-Perron et al., (1985) supra) to
generate pCGN40. A 746bp EcoRV-NaeI fragment of the
mas-5' region is replaced by an XhoI site by digesting

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pCGN40 with EcoRV and NaeI followed by ligation in the
presence of a synthetic XhoI linker DNA to create
pCGN1036. The 765bp SstI-HindIII fragment (bp 18474-
19239) of pCGN14, containing the mas-3' region, is
cloned into SstI-HindIII digested pUC18 (Norrander et
al., (1983) supra) to yield pCGN43. The HindIII site
of pCGN43 is replaced with an EcoRI site by digestion
with HindIII, blunt ending with Klenow fragment, and
ligation of synthetic EcoRI linker DNA to create
pCGN1034. The 767bp EcoRI fragment of pCGN1034 is
cloned into EcoRI-digested pCGN1036 in the orientation
that places nucleotide 19239 of the mas-3' region
proximal to the mas-5' region to create pCGN1040.
Plasmid pCGN1040 is subjected to partial digestion with
SstI, treated with T4 DNA polymerase to create blunt
ends, and ligated in the presence of synthetic XhoI
linker DNA. A clone is selected in which only the SstI
site at the junction of nucleotide 18474 and vector DNA
(constructed in pCGN43 and carried into pCGN1040) is
replaced by an XhoI site to generate pCGN1047.

Plasmid pCGN565 (see above) is digested with EcoRI
and HindIII, treated with Klenow fragment to create
blunt ends, and ligated in the presence of synthetic
XhoI linker DNA to create pCGN1003. This recreates the
EcoRI site adjacent to the XhoI linker. Plasmid
pCGN1003 is digested with EcoRI, treated with Klenow
fragment to create blunt ends, and ligated in the
presence of synthetic PstI linker DNA to create
pCGN1007. The 1.5kb XhoI fragment of pCGN1047,
containing the mas-5' region and the mas-3' region with
multiple cloning sites between, is cloned into XhoI-
digested pCGN1007 to construct pCGN1052. A portion of
the multiple cloning site of pCGN1052 is deleted by
digestion with XbaI and SstI, treated with Klenow
fragment to make blunt ends, and ligated to generate
pCGN1052ΔX5.

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The 1kb EcoRI-SmaI fragment of pCGN783 (pCGN783 is
a binary plasmid containing the left and right T-DNA
borders of A. tumefaciens (Barker et al., (1983) supra)
the gentamicin resistance gene of pPH1JI (Hirsch and
5   Beringer, (1984) supra), the Kan^r gene of Tn5 (Jorgenson
et al., (1979) supra and Wolff et al., Nuc. Acids Res.
13:355-367 (1985)) and the 3' region from transcript 7
of pTiA6 (Barker et al., (1983) supra; plasmid pCGN783
has been deposited with the ATCC in Rockville, MD, on
10   December 23, 1988 under accession number 67868),
containing the 1 ATG-Kan^r gene, is cloned into EcoRI-
SmaI digested Bluescript M13-KS (Strategene, Inc., San
Diego, CA) to create pBSKm; this plasmid contains an
M13 region allowing generation of single-stranded DNA.
15   Single-stranded DNA is generated according to the
supplier's recommendations, and in vitro mutagenesis is
performed (Adelman et al., DNA 2:183-193 (1983)) using
a synthetic oligonucleotide with the sequence
5' GAACTCCAGGACGAGGC 3' to alter a PstI site with the
20   Kan^r gene and make it undigestible, creating pCGN1534.
Plasmid pCGN1534 is digested with SmaI and ligated in
the presence of synthetic EcoRI linker DNA to generate
pCGN1535.

The 1kb EcoRI fragment of pCGN1535 is cloned into
25   EcoRI-digested pCGN1052ΔX5 to create the mas-5'-Kan^r-
mas-3' plant selectable marker cassette pCGN1536.

Construction of pCGN1541b

Plasmid pCGN565RBα2X (see below) is digested with
BglII and XhoI, and the 728bp fragment containing the
30   T-DNA right border piece and the lacZ' gene is ligated
with BglII- and XhoI-digested pCGN65ΔKX-S+K (see
below), replacing the BglII-XhoI right border fragment
of pCGN65ΔKX-S+K. The resulting plasmid, pCGN65α2X
contains both T-DNA borders and the lacZ' gene. The
35   ClaI fragment of pCGN65α2X is replaced with an XhoI
site by digesting with ClaI, blunting the ends using

Klenow fragment, and ligating with XhoI linker DNA,
resulting in plasmid pCGN65α2XX. Plasmid pCGN65α2XX is
digested with BglII and EcoRV, treated with Klenow
fragment to create blunt ends, and ligated in the
presence of BglII linker DNA, resulting in pCGN65α2XX'.
Plasmid pCGN65α2XX' is digested with BglII and ligated
with BglII-digested pCGN1538 (see below), resulting in
pCGN1541a, which contains both plasmid backbones.
Plasmid pCGN1541a is digested with XhoI and religated.
Ampicillin resistant (Amp^r), chloramphenicol sensitive
clones are chosen, which lack the pACYC184-derived
backbone, creating pCGN1541b.

Plasmid pCGN1538 is generated by digesting pBR322
with EcoRI and PvuII, treating with Klenow fragment to
generate blunt ends, and ligating with BglII linkers.
Plasmid pCGN1538 is ampicillin resistant, tetracycline
sensitive.

Construction of pCGN65ΔKX-S+K

Plasmid pCGN501 is constructed by cloning a 1.85kb
EcoRI-XhoI fragment of pTiA6 (Currier and Nester,
(1976) supra) containing bases 13362-15208 (Barker et
al., (1983) supra) of the T-DNA (right border), into
EcoRI- and SalI-digested M13mp9 (Vieira and Messing,
(1982) supra). Plasmid pCGN502 is constructed by
cloning a 1.6kb HindIII-SmaI fragment of pTiA6,
containing bases 602-2212 of the T-DNA (left border),
into HindIII- and SmaI-digested M13mp9. Plasmids
pCGN501 and pCGN502 are both digested with EcoRI and
HindIII and both T-DNA-containing fragments cloned
together into HindIII digested pUC9 (Vieira and
Messing, (1982) supra) to yield pCGN503, containing
both T-DNA border fragments. Plasmid pCGN503 is
digested with HindIII and EcoRI and the two resulting
HindIII-EcoRI fragments (containing the T-DNA borders)
are cloned into EcoRI-digested pHC79 (Hohn and Collins,
Gene 11:291-298 (1980)) to generate pCGN518. The 1.6kb

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KpnI-EcoRI fragment from pCGN518, containing the left
T-DNA border, is cloned into KpnI- and EcoRI-digested
pCGN565 to generate pCGN580. The BamHI-BglII fragment
of pCGN580 is cloned into the BamHI site of pACYC184

5   (Chang and Cohen, (1978) supra) to create pCGN51. The
1.4kb BamHI-SphI fragment of pCGN60, containing the T-
DNA right border fragment, is cloned into BamHI- and
SphI-digested pCGN51 to create pCGN65, which contains
the right and left T-DNA borders.

10   Plasmid pCGN65 is digested with KpnI and XbaI,
treated with Klenow fragment to create blunt ends, and
ligated in the presence of synthetic BglII linker DNA
to create pCGN65ΔKX. Plasmid pCGN65ΔKX is digested
with SalI, treated with Klenow fragment to create blunt

15   ends, and ligated in the presence of synthetic XhoI
linker DNA to create pCGN65ΔKX-S+X.

         <u>Construction of pCGN565RBα2X</u>

         Plasmid pCGN451 (see below) is digested with HpaI
and ligated in the presence of synthetic SphI linker

20   DNA to generate pCGN55. The XhoI-SphI fragment of
pCGN55 (bp 13800-15208, including the right border of
T-DNA (Barker et al., Gene 2:95-113 (1977)) is cloned
into SalI- and SphI-digested pUC19 (Yanisch-Perron et
al., (1985) supra) to create pCGN60. The 1.4kb

25   HindIII-BamHI fragment of pCGN60 is cloned into
HindIII- and BamHI-digested pSP64 (Promega, Inc.) to
generate pCGN1039. Plasmid pCGN1039 is digested with
SmaI and NruI (deleting bp 14273-15208; (Barker et al.,
(1977) supra)) and ligated in the presence of synthetic

30   BglII linker DNA creating pCGN1039ΔNS. The 0.47kb
EcoRI-HindIII fragment of pCGN1039ΔNS is cloned into
EcoRI- and HindIII-digested pCGN565 to create
pCGN565RB. The HindIII site of pCGN565RB is replaced
with an XhoI site by digesting with HindIII, treating

35   with Klenow fragment, and ligating in the presence of
synthetic XhoI linker DNA to create pCGN565RB-H+X.

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Plasmid pUC18 (Norrander et al., (1983) supra) is digested with HaeII to release the lacZ' fragment, treated with Klenow fragment to create blunt ends, and the lacZ'-containing fragment is ligated into

5   pCGN565RB-H+X, which had been digested with AccI and SphI and treated with Klenow fragment, in such an orientation that the lacZ' promoter is proximal to the right border fragment; this construct, pCGN565RBα2x is positive for lacZ' expression when plated on an

10   appropriate host and contains bp 13990-14273 of the right border fragment (Barker et al., (1977) supra) having deleted the AccI-SphI fragment (bp 13800-13990).

Construction of pCGN451

Plasmid pCGN451 contains an ocs5'-ocs3' cassette,

15   including the T-DNA right border, cloned into a derivative of pUC8 (Vieira and Messing, (1982) supra). The modified vector is derived by digesting pUC8 with HincII and ligating in the presence of synthetic linker DNA, creating pCGN416, and then deleting the EcoRI site

20   of pCGN416 by EcoRI digestion followed by treatment with Klenow fragment and self-ligation to create pCGN426.

The ocs5'-ocs3' cassette is created by a series of steps from DNA derived from the octopine Ti-plasmid

25   pTiA6 (Currier and Nester, (1976) supra). To generate the 5'end, which includes the T-DNA right border, an EcoRI fragment of pTiA6 (bp 13362-16202 (the numbering is by Barker et al., (1977) supra for the closely related Ti plasmid pTi15955)) is removed from pVK232

30   (Knauf and Nester, (1982) supra) by EcoRI digestion and cloned into EcoRI digested pACYC184 (Chang and Cohen, (1978) supra) to generate pCGN15.

The 2.4kb BamHI-EcoRI fragment (bp 13774-16202) of pCGN15 is cloned into EcoRI- and BamHI-digested pBR322

35   (Bolivar et al., (1977) supra) to yield pCGN429. The 412bp EcoRI-BamHI fragment (bp 13362-13772) of pCGN15

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is cloned into EcoRI- and BamHI-digested pBR322 to
yield pCGN407. The cut-down promoter fragment is
obtained by digesting pCGN407 with XmnI (bp 13512),
followed by resection with Bal31 exonuclease, ligation
of synthetic EcoRI linkers, and digestion with BamHI.
Resulting fragments of approximately 130bp are gel
purified and cloned into M13mp9 (Vieira and Messing,
(1982) supra) and sequenced. A clone, I-4, in which
the EcoRI linker has been inserted at position 13642
between the transcription initiation point and the
translation initiation codon, is identified by
comparison with the sequence of de Greve et al., J.
Mol. Appl. Genet. 1:499-512 (1982). The EcoRI cleavage
site is at position 13639, downstream from the mRNA
start site. The 141bp EcoRI-BamHI fragment of I-4,
containing the cut-down promoter, is cloned into EcoRI-
and BamHI-digested pBR322 to create pCGN428. The 141bp
EcoRI-BamHI promoter piece from pCGN428 and the 2.5kb
EcoRI-BamHI ocs5' piece from pCGN429 are cloned
together into EcoRI-digested pUC19 (Vieira and Messing,
(1982) supra) to generate pCGN442, reconstructing the
ocs upstream region with a cut-down promoter section.

      To generate the ocs3' end, the HindIII fragment of
pLB41 (D. Figurski, University of California, San
Diego) containing the gentamicin resistance gene is
cloned into HindIII-digested pACYC184 (Chang and Cohen,
(1978) supra) to create pCGN413b. The 4.7kb BamHI
fragment of pTiA6 (Currier and Nester, (1976) supra),
containing the ocs3' region, is cloned into BamHI
digested pBR325 (Bolivar, Gene 4:121-136 (1978)) to
create 33c-19. The SmaI site at position 11207 (Barker
et al., (1977) supra) of 33c-19 is converted to an XhoI
site using a synthetic XhoI linker, generating
pCGN401.2. The 3.8kb BamHI-EcoRI fragment of pCGN401.2
is cloned into BamHI- and EcoRI-digested pCGN413b to
create pCGN419.

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The ocs5'-ocs3' cassette is generated by cloning the 2.64kb EcoRI fragment of pCGN442, containing the 5' region, into EcoRI-digested pCGN419 to create pCGN446. The 3.1kb XhoI fragment of pCGN446, having the ocs5' region (bp 13639-15208) and ocs3' region (bp 11207-12823), is cloned into the XhoI site of pCGN426 to create pCGN451.

Example 6: Synthesis of the Thionin Signal Peptide/Magainin-1 DNA Sequence

The DNA sequence for expression and transport of the AMP magainin M1 is assembled from oligonucleotides using the Klenow fragment of DNA polymerase and the polymerase chain reaction (PCR) technique, as described in U.S. Patent Nos. 4,683,195 and 4,683,202.

The DNA sequence for expression and transport of M1 consists of six non-specific base pairs followed by a BamHI site, then the DNA sequence encoding the putative signal peptide from a barley leaf-specific thionin (to allow for transport out of the cell) fused in-frame to the mature magainin M1 coding region, an Asp718 site, and six more non-specific base pairs. The BamHI and Asp718 sites allow the gene to be cloned into the double 35S expression cassette pCGN1431 (described above); the non-specific bases on the ends are employed to provide double-stranded helix structure on both sides of the restriction sites, which most restriction enzymes require for activity. The codons are selected in the design of this DNA sequence based on codon preferences for dicotyledonous plants (Murray et al., (1989) supra) to allow maximal translation rate in the target plants of the present example (Brassica sp.). Codon selection for other target plant species will be made as a matter of ordinary skill.

The DNA sequence which encodes the putative signal peptide of barley leaf thionin is 127bp in length. Two

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overlapping oligonucleotides were designed which, when
annealed and extended with Klenow fragment, comprise
the entire sequence: "SIG-T" is 73 "sense" strand
bases, consisting of bases of 1-73 of the DNA sequence
5   encoding the thionin signal peptide (as described by
Gausing, (1987) supra):

    "SIG-T":
    5'-CATATCTCATTCTACTTGAGAAATTAAGGCCAACCA
GCCAACCATGGCAACCAACAAGTCTATTAAGTCCGTG-3'

10   "SIG-B" is 74 "anti-sense" strand bases, consisting of
bases 127-54 of the DNA sequence encoding the signal
peptide (Gausing, (1987) supra):

    "SIG-B":
    5'-GGCTTCAACTTGGACCTGCTCAAGAACCAAACCCAA
15   TATAAGAACACAAATAACCACGGACTTAATAGACTTGT-3'

Bases 54-73 of "SIG-T" and "SIG-B" overlap as
complementary strands, and when allowed to anneal they
can be extended with Klenow fragment to yield a full
length double-stranded DNA fragment of 127bp.
20   In practice, only a relatively small fraction of
the polymerase reaction products will comprise the full
127 base pairs, since the synthesis of long oligomers
(>40 bases) is not highly efficient. By employing the
PCR as described below, the full length molecules can
25   be selectively amplified, reducing the quantity of
short fragments to an insignificant level.
The M1 coding region is 72bp in length. It was
constructed in a manner similar to the signal peptide
DNA sequence, with "MAG-T" being "sense" bases 1-45,
30   and "MAG-B" being "antisense" bases 72-28, based on
magainin M1 DNA sequence as reported by Zasloff (Proc.
Natl. Acad. Sci. 84:5449-5453 (1987)).

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"MAG-T":

5'-GGAATTGGTAAGTTCTTGCACTCTGCTGGTAAGTTCGGTAAGGCT-3'

"MAG-B":

5'-TTAAGACTTCATAATCTCTCCAACAAAAGCCTTACCGAACTTACC-3'

These oligonucleotides were annealed and repaired with the Klenow fragment as described above.

Four smaller oligonucleotides were synthesized in order to allow fusion of the signal sequence and the M1 sequence, facilitate addition of the restriction sites, and allow for amplification of full-length fragments from the above polymerase reactions:

"SIG-L" is a 33 base oligonucleotide consisting of six non-specific bases, six bases comprising the BamHI site, and the first 21 "sense" bases (1-21) of the signal peptide DNA sequence:

5'-CGAGTTGGATCCVCATATCTCATTCTACTTGAGA-3'

"MAG-R" is a 33 base oligonucleotide consisting of six non-specific bases, six bases comprising the AspI site, and the last 12 "anti-sense" bases (199-178) of the M1 sequence:

5'-TGAGTTGGTACCTTAAGACTTCATAATCTCTCC-3'

"F-SM-T" is a 42 base oligonucleotide consisting of the last 21 "sense" bases of the signal peptide and the first 21 "sense" bases of M1:

5'-GAGCAGGTCCAAGTTGAAGCCGGAATTGGTAAGTTCTTGCAC-3'

"F-SM-B" is a 42 base pair of oligonucleotide complementary to "F-SM-T" consisting of the first 21 "anti-sense" bases of M1 and the last 21 "anti-sense" bases of the signal peptide:

5'-GTGCAAGAACTTACCAATTCCGGCTTCAACTTGGACCTGCTC-3'

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When "SIG-L" and "F-SM-B" are used as primers in
the PCR with the repaired signal peptide DNA fragment
as template, the resulting product comprises six non-
specific base pairs and the BamHI site, the signal
peptide DNA sequence, and 21 base pairs of the M1 DNA
sequence. PCR is performed using a DNA Thermal Cycler
machine (Perkin Elmer Cetus; Emeryville, CA) as
described above.

When "MAG-R" and "F-SM-T" are used as PCR primers
with the repaired M1 DNA fragment as template, the
resulting product comprises the 21 base pairs of the
signal peptide DNA sequence, the M1 DNA sequence, the
Asp718 site, and six non-specific base pairs. Each of
these reactions selectively amplifies only the full-
length or near full-length fragments from the initial
repair reactions, permitting the use of crude
oligonucleotides in all steps, and resulting in a great
savings of time and manpower.

These two double-stranded products share a 42bp
overlap which defines the fusion junction for the
thionin signal sequence and the M1 sequence. Upon
strand melting (such as occurs during the PCR), a
portion of each type will reanneal with an overlapping
strand of the other, forming a complex which can repair
into the full-length DNA sequence. When the two
fragments are mixed in a 1:1 ratio, and amplified in
the PCR using "SIG-L" and "MAG-A" as primers, the
resulting product is the full-length sequence with the
appropriate restriction sites attached.

The actual assembly of the DNA signal/magainin
sequence is carried out in several steps. One nmol
each of "MAG-T" and "MAG-B" (for the M1 region; "SIG-
T" and "SIG-B" are used for the signal region) are
combined with 1 μl 100XTE in a total volume of 100μl.
This mixture is placed in a DNA Thermal Cycler (Perkin
Elmer Cetus; Emeryville, CA) and the DNA strands are

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melted and annealed for 30 minutes in a cooling
gradient from 80°C to 37°C followed by 15 minutes at
37°C. Ten μl of this reaction is treated with Klenow
fragment in a 50μl reaction volume to repair the
strands. One μl of the Klenow fragment reaction is
used in a PCR reaction with "F-SM-T" and "MAG-A"
("F-SM-B" and "SIG-L") as primers to add the fusion
region. The PCR conditions are as follows: 94°C for 1
min., 55°C for 2 min., 72°C for 3 min., for a total of
25 cycles.

The products from each PCR reaction (resulting in
the M1 region and the signal region) are then used as
template in another PCR reaction: Samples from each
reaction are combined in a one-to-one ratio and
subjected to PCR using primers "SIG-L" and "MAG-R" at
94°C for 1 min., 50°C for 2 min., 72°C for 3 min., for
a total of 25 cycles. PCR products are then digested
with Asp718 and BamHI and cloned into pCGN1431.

Following digestion with BamHI and Asp718, the
thionin signal/M1 DNA sequence may be cloned into
BamHI- and Asp718-digested pCGN1431. The expression
cassette is then cloned into a binary vector, such as
PCGN1557 (described above), and the resulting construct
is introduced into Agrobacterium tumefaciens by
transformation. The transformed A. tumefaciens is then
used to generate transformed plants, as described in
Example 7.

Example 7: Brassica Transformation

Seeds of Brassica napus cv. Westar are soaked in
95% ethanol for 2 min., surface sterilized in a 1.0%
solution of sodium hypochlorite containing a drop of
Tween®20 for 45 min., and rinsed three times in
sterile, distilled water.

Treated seeds are then plated in Magenta boxes
(Magenta Corp., Chicago, Illinois) with 1/10th

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concentration of Murashige minimal organics medium
(Gibco) supplemented with pyridoxine (50μg/l),
nicotinic acid (50μg/l), glycine (200μg/l), and 0.6%
Phytagar (Gibco) at pH 5.8. The seeds are then
5   germinated in a culture room at 22°C in a 16 hour
photoperiod with cool fluorescent and red light of
intensity approximately 65μ Einsteins per square meter
per second ($\mu Em^{-2}s^{-1}$)

Hypocotyls are excised from the resultant 6 day
10   old seedlings, cut into pieces approximately 4mm in
length, and plated on feeder plates (Horsch, et al.,
Science 227:1224-1231 (1985)).

Feeder plates are prepared one day before use by
plating 0.5ml of a tobacco suspension culture onto a
15   petri plate (100x25mm) containing about 30ml MS salt
base (Carolina Biological), 100mg/l inositol, 1.3mg/l
thiamine-HCl, 200mg KH_2PO_4 with 3% sucrose, 1.0mg/l 2,4-
dichlorophenoxyacetic acid (2,4-D), 0.6% Phytagar, and
pH adjusted to 5.8 prior to autoclaving (termed MS0/1/0
20   medium). A sterile filter paper disc (Whatman® 3mm) is
placed on top of the feeder layer prior to use.

Tobacco suspension cultures are subcultured weekly
by transfer of 10ml of culture into 100ml fresh MS
medium as described for the feeder plates, but with
25   0.2mg/l 2,4-D and 0.1mg/l Kinetin.

In embodiments where feeder cells are not used,
hypocotyl explants are cut and placed onto a filter
paper disc on top of MS0/1/0 medium. All hypocotyl
explants are preincubated on feeder plates for 24 hours
30   at 22°C in continuous light of intensity in the range
of $30\mu Em^{-2}s^{-1}$ to $65\mu Em^{-2}s^{-1}$.

Single colonies of A. tumefaciens strain EHA 101,
containing a binary plasmid, are transferred to 5ml
MG/L broth (consisting of 5g mannitol, 1g L-glutamic
35   acid or 1.15g sodium glutamate, 0.5g KH_2PO_4, 0.10g NaCl,
0.10g $MgSO_4 \cdot 7H_2O$, 1μg biotin, 5g tryptone, 2.5g yeast

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extract (per liter, pH adjusted to 7.0)), and grown overnight at 30°C.

Hypocotyl explants are then immersed in 7-12ml MG/L broth with bacteria diluted to 1×10^8 bacteria/ml and after 10-25 min. are transferred onto feeder plates. After 48 hours of co-incubation with Agrobacterium, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium (B5 salts and vitamins supplemented with 1mg/l 2,4-D and 3% sucrose - Gamborg, et al., Exp. Cell Res. 50:151-158 (1968)) which contains filter sterilized carbenicillin (500mg/l, added after autoclaving) and 25mg/l kanamycin sulfate (Boehringer Mannheim).

After 3 days in culture at 65-75 $\mu Em^{-2}s^{-1}$ continuous light, hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3mg/l benzylaminopurine, 1mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500mg/l) and kanamycin sulfate (25mg/l). Hypocotyl explants were subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, 300mg/l carbenicillin, 50mg/l kanamycin sulfate and 0.6% Phytagar. After 2-4 weeks, shoots which remain green were cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2mg/l indolebutyric acid, 50mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for NPTII activity.

In accordance with one aspect of the subject

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invention, plant-associated pathogen resistance can be imparted to susceptible hosts to provide for enhanced protection of the host crop. In addition, DNA sequences are provided which can be used for producing
5   selected AMPs, for example, in methods of the present invention. Furthermore, a method is provided for inhibiting plant associated pathogens by applying selected AMPs. Thus, plants can be grown which will be less susceptible to a variety of plant-associated
10   pathogens in order to greatly enhance the utility of the crop plants and allow their growth in regions wherein plant associated pathogen infestation is endemic.

All publications and patent applications cited in
15   this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been
20   described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teaching of this invention that certain changes and modifications may be made thereto
25   without departing from the spirit or scope of the appended claims.

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Claims

1.   A method of limiting growth of a plant-
associated pathogen comprising contacting a plant-
associated pathogen with a plant-associated pathogen-
inhibiting amount of an animal-derived anti-microbial
5   peptide under growth limiting conditions and allowing
said anti-microbial peptide to act upon said plant-
associated pathogen thereby limiting growth by said
plant-associated pathogen.

10   2.   The method of Claim 1 wherein said animal-
derived antimicrobial peptide is selected from the
family of magainins.

3.   The method of Claim 1 wherein said animal-
derived antimicrobial peptide is selected from the
15   family of defensins.

4.   The method of Claim 1 wherein said plant
associated pathogen is a fungus.

5.   The method of Claim 4 wherein said fungus is
selected from the group consisting of
20   Plasmodiophormycetes species, Chytridiomycetes species,
Zygomycetes species, Oomycetes species, Ascomycetes
species, Basidiomycetes species and Deuteromycetes
species.

6.   The method of Claim 5 wherein said fungus is
25   selected from the group consisting of Fusarium
oxysporum, Pythium ultimum, and Verticillium albo-
atrium.

7.   The method of Claim 1 wherein said plant
associated pathogen is a bacterium.

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8.   The method of Claim 6 wherein said bacterium
is selected from the group consisting of Agrobacterium
species, Corynebacterium species, Erwinia species,
Pseudomonas species, Xanthomonas species, Streptomyces
5   species and Rhizobium species.

9.   The method of Claim 7 wherein said bacterium
is selected from the group consisting of Erwinia
carotovora and Pseudomonas syringae, and Xanthamonas
campestris.

10   10. The method of Claim 1 wherein said growth is
inhibited.

11. The method of Claim 1 wherein said growth is
substantially slowed.

12. A DNA construct comprising a transcriptional
15   initiation region functional in plants joined at its 3'
terminus to the 5' terminus of a DNA sequence which
codes for at least a portion of a signal peptide which
is secreted from a plant cell and an animal-derived
anti-microbial peptide.

20   13. The DNA construct of Claim 12 wherein said
animal-derived antimicrobial peptide is selected from
the family of magainins.

14. The DNA construct of Claim 13 wherein said
DNA sequence codes for a peptide selected from the
25   group consisting of:
          gly-ile-gly-lys-phe-leu-his-ser-ala-gly-lys-phe-
          gly-lys-ala-phe-val-gly-glu-ile-met-lys-ser,

          gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-
          gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

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ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-
lys-ala-phe-val-gly-glu-ile-met-asn-ser,

gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-
lys-ala-phe-val-gly-glu-ile-met-asn-ser,

lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-
ala-phe-val-gly-glu-ile-met-asn-ser,

phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-
phe-val-gly-glu-ile-met-asn-ser,

leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-
val-gly-glu-ile-met-asn-ser, and

gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-
phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn.

15. The DNA construct of Claim 12 wherein said animal-derived antimicrobial peptide is selected from the family of defensins.

16. The DNA construct of Claim 15 wherein said DNA sequence codes for a peptide selected from the group consisting of:

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-
pro-arg-glu-arg-arg-ala-gly-phe-cys-arg-ile-
arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-
pro-leu-glu-arg-arg-ala-gly-phe-cys-arg-ile-
arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

gly-ile-cys-ala-cys-arg-arg-arg-phe-cys-pro-
asn-ser-glu-arg-phe-ser-gly-tyr-cys-arg-val-
asn-gly-ala-arg-tyr-val-arg-cys-cys-ser-arg-arg,

gly-arg-cys-val-cys-arg-lys-gln-leu-leu-cys-ser-
tyr-arg-glu-arg-arg-ile-gly-asp-cys-lys-ile-
arg-gly-val-arg-phe-pro-phe-cys-cys-pro-arg,

val-ser-cys-thr-cys-arg-arg-phe-ser-cys-gly-
phe-gly-glu-arg-ala-ser-gly-ser-cys-thr-val-
asn-gly-val-arg-his-thr-leu-cys-cys-arg-arg, and

val-phe-cys-thr-cys-arg-gly-phe-leu-cys-gly-
ser-gly-glu-arg-ala-ser-gly-ser-cys-thr-ile-
asn-gly-val-arg-his-thr-leu-cys-cys-arg.

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17. The DNA construct of Claim 12 wherein said plant associated pathogen is a fungus.

18. The DNA construct of Claim 17 wherein said fungus is selected from the group consisting of Plasmodiophormycetes species, Chytridiomycetes species, Zygomycetes species, Oomycetes species, Ascomycetes species, Basidiomycetes species, and Deuteromycetes species.

19. The DNA construct of Claim 18 wherein said fungus is selected from the group consisting of Fusarium oxysporum, Pythium ultimum, and Verticillium albo-atrium.

20. The DNA construct of Claim 12 wherein said plant associated pathogen is a bacterium.

21. The DNA construct of Claim 20 wherein said bacterium is selected from the group consisting of Agrobacterium species, Corynebacterium species, Erwinia species, Pseudomonas species, Xanthomonas species, Streptomyces species and Rhizobium species.

22. The DNA construct of Claim 21 wherein said bacterium is selected from the group consisting of Erwinia carotovora and Pseudomonas syringae, and Xanthamonas campestris.

23. A method for improving disease resistance in plants which comprises:
    modifying a plant with a DNA construct comprising a transcriptional initiation region functional in plants joined at its 3' terminus to the 5' terminus of a DNA sequence which codes for a structural gene for at least a portion of a

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signal peptide which is secreted from a plant cell
and an animal-derived anti-microbial peptide, said
structural gene being under the transcriptional
control of said plant host and being capable, in
5   said plant host, of producing a plant-associated
pathogen-inhibiting amount of said anti-microbial
peptide under growth limiting conditions.

24.   The method of Claim 23 wherein said animal-
derived antimicrobial peptide is selected from the
10   family of magainins.

25.   The method of Claim 24 wherein said
structural gene codes for a peptide selected from the
group consisting of:

15   gly-ile-gly-lys-phe-leu-his-ser-ala-gly-lys-phe-
          gly-lys-ala-phe-val-gly-glu-ile-met-lys-ser,

          gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-
          gly-lys-ala-phe-val-gly-glu-ile-met-asn-ser,

          ile-gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-
          lys-ala-phe-val-gly-glu-ile-met-asn-ser,

20   gly-lys-phe-leu-his-ser-ala-lys-lys-phe-gly-
          lys-ala-phe-val-gly-glu-ile-met-asn-ser,

          lys-phe-leu-his-ser-ala-lys-lys-phe-gly-lys-
          ala-phe-val-gly-glu-ile-met-asn-ser,

          phe-leu-his-ser-ala-lys-lys-phe-gly-lys-ala-
25   phe-val-gly-glu-ile-met-asn-ser,

          leu-his-ser-ala-lys-lys-phe-gly-lys-ala-phe-
          val-gly-glu-ile-met-asn-ser, and

          gly-ile-gly-lys-phe-leu-his-ser-ala-lys-lys-
          phe-gly-lys-ala-phe-val-gly-glu-ile-met-asn.

30   26.   The DNA construct of Claim 23 wherein said
animal-derived antimicrobial peptide is selected from
the family of defensins.

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27. The method of Claim 26 wherein said
structural gene codes for a peptide selected from the
group consisting of:

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-
pro-arg-glu-arg-arg-ala-gly-phe-cys-arg-ile-
arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

val-val-cys-ala-cys-arg-arg-ala-leu-cys-leu-
pro-leu-glu-arg-arg-ala-gly-phe-cys-arg-ile-
arg-gly-arg-ile-his-pro-leu-cys-cys-arg-arg,

gly-ile-cys-ala-cys-arg-arg-arg-phe-cys-pro-
asn-ser-glu-arg-phe-ser-gly-tyr-cys-arg-val-
asn-gly-ala-arg-tyr-val-arg-cys-cys-ser-arg-arg,

gly-arg-cys-val-cys-arg-lys-gln-leu-leu-cys-ser-
tyr-arg-glu-arg-arg-ile-gly-asp-cys-lys-ile-
arg-gly-val-arg-phe-pro-phe-cys-cys-pro-arg,

val-ser-cys-thr-cys-arg-arg-phe-ser-cys-gly-
phe-gly-glu-arg-ala-ser-gly-ser-cys-thr-val-
asn-gly-val-arg-his-thr-leu-cys-cys-arg-arg, and

val-phe-cys-thr-cys-arg-gly-phe-leu-cys-gly-
ser-gly-glu-arg-ala-ser-gly-ser-cys-thr-ile-
asn-gly-val-arg-his-thr-leu-cys-cys-arg.

28. The method of Claim 23 wherein said plant
associated pathogen is a fungus.

29. The method of Claim 28 wherein said fungus is
selected from the group consisting of
Plasmodiophormycetes species, Chytridiomycetes species,
Zygomycetes species, Oomycetes species, Ascomycetes
species, Basidiomycetes species and Deuteromycetes
species.

30. The method of Claim 29 wherein said fungus is
selected from the group consisting of Fusarium
oxysporum, Pythium ultimum, and Verticillium albo-
atrium.

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31. The method of Claim 23 wherein said plant associated pathogen is a bacterium.

32. The method of Claim 31 wherein said bacterium is selected from the group consisting of Agrobacterium species, Corynebacterium species, Erwinia species, Pseudomonas species, Xanthomonas species, Streptomyces species and Rhizobium species.

33. The method of Claim 32 wherein said bacterium is selected from the group consisting of Erwinia carotovora and Pseudomonas syringae, and Xanthamonas campestris.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02; C07K 7/10; C12N 15/12

U.S. CL.: 435/172.1; 514/12, 13; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴	
Classification System ₁	Classification Symbols
US	435/172.1; 514/12,13 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	US, A, 4,543,252 (LEHRER ET AL.) 24 September 1985 See entire document.	1-11
X	US, A, 4,659,692 (LEHRER ET AL.) 21 April 1987 See entire document.	1-11
X	US, A, 4,810,777 (ZASLOFF) 07 March 1989 See entire document.	1-11
Y	BioEssays, "Increasing Bacterial Disease Resistance In Plants Utilizing Antibacterial Genes From Insects" Volume 6, No. 6, pp 263-270, (JAYNES ET AL.) June 1987, See entire document.	12-33
Y,P	Science, "An Assay For Circulating Antibodies To A Major Etiologic Virus Of Human Non-A, Non-B Hepatitis", Vol. 244, pp362-364, (KUO ET AL.) 21 April 1989.	12-33

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²
02 JULY 1990	02 AUG 1990
International Searching Authority ₁	Signature of Authorized Officer ²⁰
ISA/US	LESTER L. LEE

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| Y | Science, "Inheretance of Functional Foreign Genes in Plants", Vol. 223, pp 496-498, (HORSCH ET AL.), January 1984, pp 496-498, See entire document. | 12-33 |
| Y,P | Science, "Isolation of a cDNA Clone Derived From a Blood-Borne Non-A, Non-B Viral Hepatitis Genome", Vol. 244, pp 359-362, (CHOO ET AL.) 21 April 1989. | 12-33 |

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3.☐ Claim numbers ...., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [1]

This International Searching Authority found multiple inventions in this International application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)